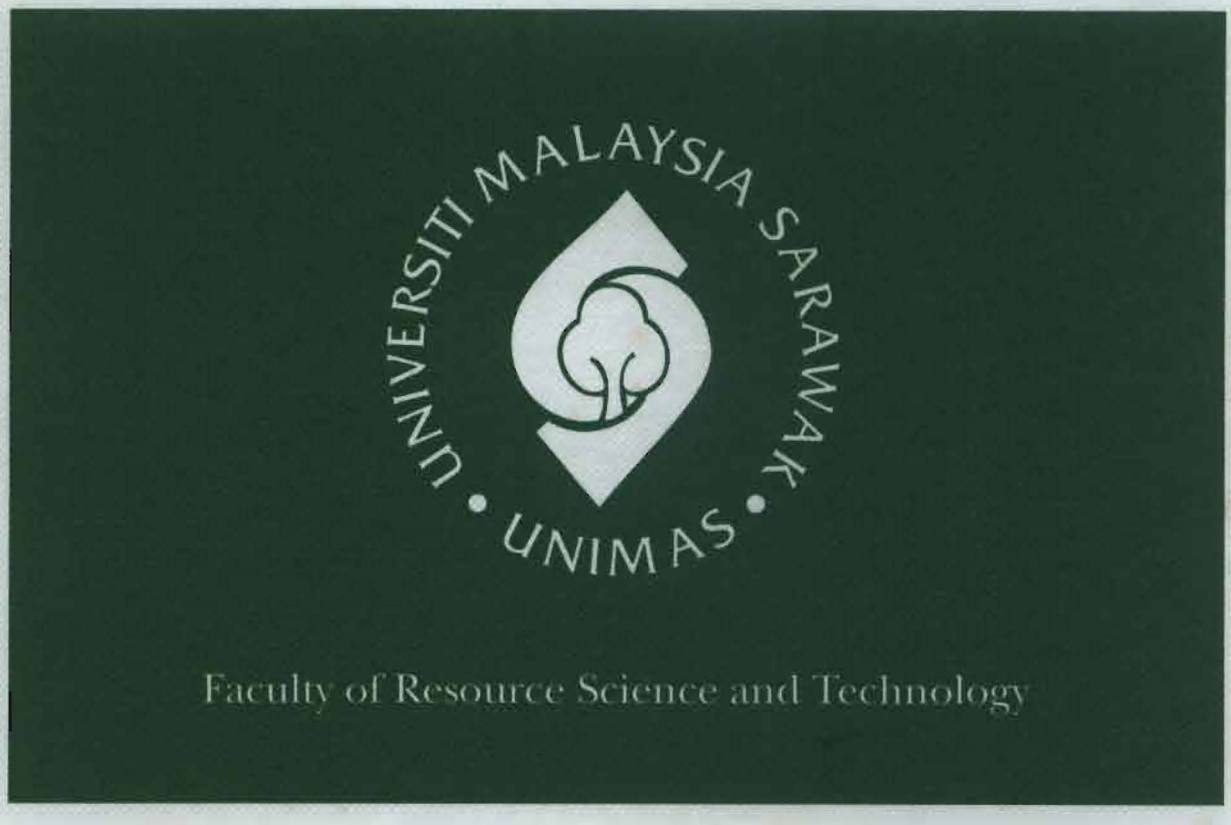

ISOLATION AND CHARACTERIZATION OF OIL DEGRADING BACTERIA FROM A LOCAL ECOLOGICAL SANITATION (ECOSAN) SYSTEM

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ABSTRACT

Eight bacterial isolates (B1, B2, B3, B4, B5, B6, B7 and B8) were isolated from the oil and grease trap of a Grey Water Treatment System (ECOSAN System) in Kenanga College. Four of the bacterial isolates were identified based on API 20E system as follows: B2 – *Chromobacterium violeceum*, B4 – *Pseudomonas aeruginosa*, B7 ,– *Pseudomonas fluorescent/putida* and B8 – *Klebsiella pneumonia pneumonia* while three of them were identified based on 16S rRNA sequencing as follows: B3 – *Bacillus sp.* CNJ782 PL04, B5 – *Pseudomonas aeruginosa* Strain PD100 and B6 – *Pseudomonas aeruginosa* strain R11. However, B1 was unidentifiable. All the eight isolates were positively screened as oil degrading bacteria by using Rhodamine B agar. B4 can be concluded as having the highest degradation activity when incubated with both 1% and 2% Tween 20 and Tween 80 compared to the other bacterial isolates.

Key words: Bacterial isolates, oil degrading bacteria, degradation activity, grey water treatment

ABSTRAK

Lapan jenis bakteria (B1, B2, B3, B4, B5, B6, B7 dan B8) telah berjaya dipencilkan daripada perangkap minyak di sebuah Grey Water System (ECOSAN System) di Kolej Kenanga. Empat daripad bakteria tersebut dikenalpasti berdasarkan sistem API 20E sebagai: B2 – Chromobacterium violeceum, ,B4 – Pseudomonas aeruginosa, , B7 – Pseudomonas fluorescent/putida dan B8 – Klebsiella pneumonia pneumoni manakala tiga lagi dikenlpasti berdasarkan 16S rRNA sequencing sebagai: B3 – Bacillus sp. CNJ782 PL04 B5 – Pseudomonas aeruginosa Strain PD100 dan B6 – Pseudomonas aeruginosa strain R11. Kesemua lapan bakteria tersebut telah menunjukkan keputusan yang positif apabila diuji dengan agar Rhodamine B. B4 mempamirkan aktiviti tertinggi dalam penguraian kesemua 1% and 2% Tween 20 dan Tween 80 jika dibandingkan dengan bakteria-bakteria lain.

Kata kunci: Bakteria yang dipencilkan, bakteria yang menguraikan minyak, aktiviti penguraian, rawatan grey water

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1.0 Introduction:

Wastewater from food processing plants, restaurants (Yumoto *et al.*, 2003), slaughterhouses, dairy and meat packing industries as well as domestic wastewater contain high amount of fats and oils from plants and animal sources (El-Bestawy *et al.*, 2005). If the wastewater is not subjected to proper treatment, the oil wastes will eventually reach the natural environments such as land and rivers, which usually can be seen as tin coloured films on water surfaces. The presence of oil films on water surfaces will prevent oxygen diffusion into water which will lead to a problem that may contribute to the destruction of many forms of aquatic flora and fauna.

Oils and fats present in wastewater are difficult to remove and degrade as they are unable to dissolve in water (Matsumiya *et al.*, 2007). Therefore, it is essential that the potential environmental hazard from oil pollution is fully appreciated and proper treatment techniques are utilized in order to minimize the risk of oil pollution. One of the solutions to oil waste problems is to treat the wastewater using ECOSAN (Ecological Sanitation) system (WASTE – advisers on urban environment and development, 2005). Even with the availability of this wastewater treatment system, the presence of high amount of oil waste in the wastewater may contribute to problems in the system itself due to the difficulty of oil to dissolve in water. Aggregates formed by oil droplets and other particles that are present in grey water may bring about obstruction to water drainage and eventually lead to the clogging of wastewater pipes.

Physical separation of lipids by using grease trap in wastewater treatment system may reduce the clogging of wastewater pipes. The physical separation method is effective for removing most lipids from wastewater, yet the separated lipids have to be incinerated

or dumped in landfills (Matsumiya *et al.*, 2007). Incineration of lipids with heavy oils and landfill dumping may be problematic to the environment. Moreover, the grease traps and separated lipids emit an offensive adour. In addition, these separation processes result in high maintenance costs and space limitation for equipment in small food manufacturers and normal domestic kitchen (Takeno *et al.*, 2005). Thus, correct treatment of waste oil together with organic matter from kitchen and restaurant wastewater is very important for protecting the local environment.

Therefore, a more effective way of removing lipid waste from the wastewater system has to be designed. It is preferable that the process is done through natural ways as what comes from the nature should be returned to the nature. Many reports have described the utilization of lipolytic enzymes in wastewater treatment. A good source of large scale production of lipase is microorganisms (Hasanuzzaman *et al.*, 2004). In recent years, concerns on bioremediation of lipid wastes prior to disposal into the environment have increased. Suzuki *et al.* (2001) stated that oil wastes are removed from the wastewater through biological approach, namely, the microbial or enzymatic degradation. This can be done by the addition of microbial lipase into the wastewater containing high amount of oil wastes. This method may lead to more efficient oils or fats degradation in wastewater.

Microbial functions have been investigated with respect to their use in treating lipid-containing wastewater. In order to improve the biodegradation of lipids in wastewater, several microorganisms such as *Pseudomonas aeruginosa, Bacillus sp* and yeasts have been studied for their lipid degradation ability on a laboratory scale (Matsumiya *et al.*, 2007). Recently, *Bacillus subtillis BN 1001* (BN Clean) has been

developed into an efficient industrial lipid containing wastewater treatment system. However, these strains do not always show their lipid degrading ability due to seasonal changes in temperature, do not degrade various types of lipid, and negatively affected by environmental bacteria in open wastewater treatment system (Matsumiya *et al.*, 2007). Thus the isolation and identification of new bacterial strains with the ability to produce lipases is very crucial, especially indigenous bacterial species present in environment such as wastewater system.

As different types of industrial application or bioremediation processes (wastewater treatment) requires lipases with specific properties, therefore additional lipases from various types of microorganisms from the environment that could be used in new applications continue to garner interest and the isolation of microorganisms with such properties are very crucial. In this research, microorganisms with the ability to degrade oil wastes will be isolated from the grey water system itself as it is believed that certain indigenous oil degrading microbes is readily available in the water containing oil waste in the system. If oil degrading microbes are successfully isolated from the system, the isolated microbes could be cultivated in the laboratory for introduction into the grey water treatment system in the future in order to enhance oil degradation rate. Thus problems derived from the accumulation of oil wastes in the grey water treatment system could be solved.

The objectives of this research are:

1. to isolate and characterize the microorganisms with the ability to degrade oil wastes from oil and grease trap of a local ECOSAN Grey water Treatment Facility (GTF), at Kenanga College.
2. to asses the oil degradation ability of the isolated oil degrading bacteria.

2.0 Literature Review:

2.1 ECOSAN Systems

WASTE – advisers on urban environment and development (2005) reported that over one billion people around the world lack access to safe drinking water and more than 2.4 billion do not have access to hygienic sanitary facilities. Consequently, every year more than three million people die from water related diseases. The Millennium Development Goal (MDG) set by UNDP in Johannesburg in 2000, target to halve the proportion of people without sustainable access to safe drinking water and sanitation by the year 2015.

Conventional sewerage and wastewater treatment systems are unable to overcome this problem as the methods require high construction and maintenance costs as well as relying upon a dependable water supply in order to function correctly. Apart from that, the existing network of sewerage and wastewater treatment systems in developing countries, primarily serve only the rich. People have also begun to recognize that the sewerage systems do not allow for recovery and recycling of valuable nutrients into the food production loop. As a result, the utilization of alternative sanitation options such as the ECOSAN system continues to garner interest in overcoming the limitations of the conventional sanitation system.

ECOSAN, short for Ecological Sanitation, seeks to address the issues of modern sanitation systems, which are environmentally destructive. Ecological sanitation systems include the collection – storage – transfer & transport – treatment – and the resource management and reuse stage (UNESCO/IPH, 2006). Ideally, ECOSAN systems enable an almost complete recovery of all nutrients, trace elements and energy contained in

household wastewater and organic waste and their reuse in agriculture. An essential step in this process is the appropriate treatment and handling of materials during the entire treatment and reuse process to ensure a sufficient sanitisation of the excrement and the protection of the public health. Therefore, ECOSAN systems not only control the direct hygienic risks to the population but also protect the natural environment.

In practice a frequently applied strategy in ecological sanitation management services is to separately collect and treat faeces, urine and grey water, which minimizes the volume of valuable drinking water needed to flush away excreta. This has other important advantages as the different fractions have different characteristics and can be treated more easily according to the specific reuse requirements. Although the recovered material from human excreta is predominantly used in agriculture, urine as direct fertiliser and faeces as organic matter for soil improvement, the reuse options within ecological sanitation are not limited to agriculture only, especially considering grey water.

Other reuse options include the domestic reuse of grey water, following suitable treatment, for example for flushing toilets, or possibly its use as service water in industry, or its use to recharge groundwater. Rainwater use could also be incorporated into this, with rainwater possibly being treated and being used for drinking water. Organic material can also be recovered to generate biogas, or perhaps even as a general soil amendment (UNESCO/IPH, 2006). Biogas production allows to recuperate the energy contained in liquid and solid household wastes, and to put it to an array of uses, such as cooking, electricity generation, and heating purposes or even for industrial use.

Among the countries in the world, Australia is one of the countries which had widely adopted the ECOSAN systems. Among the ECOSAN systems which had been used in Australia includes ECOSAN toilet and grey water treatment system (UNESCO/IPH, 2006). While in Mexico, composting latrines were being used in a low-income peri-urban community utilizing US-EPA guidelines for the end product (Rautanen and Viskari *et al.*, 2006). Users were satisfied with their latrines, double vault gave a clear separation & more room for urinal. Dehydrating system was consistently the better choice over the biodegrading system in this area (very hot desert area, good for dehydration).

Fiji is another example of countries which had been adopted the ECOSAN systems. Lalati Resort, Beqa Island, Fiji had been successful in utilizing the ECOSAN systems in the treatment of grey water and black water discharged from the resort (ECOWATERS PROJECTS, 2000). A system for upscale resort with low cost, locally available materials and with flush toilets (which could be maintained by the resort staff) had been designed. The system consists of a rooftop rainwater collection system, microflush toilet (flushed with rainwater and draining to a composting reactor), and a wastewater garden planted with local flowering thirsty plants (functions to collect and treat grey water). The owners of the resort like the fact that the system does not pollute the coastline and that it is maintained by the resort staff. They also enjoyed significant cost savings over a conventional septic system. The resort's guests said that they appreciate the fact that their accommodations employ such an ecological friendly system.

2.2 Grey water

Grey water constitutes the major part of household water (Ottosson and Stenstrom, 2003). Grey water is used water from home consisting of water from baths, showers, sinks, dishwashers, and washing machines with the exception of water from toilet. Grey water contains what is washed down the drain, and therefore varies from one household to another. Grey water may contain high salt concentrations resulting from powdered laundry detergent which contains phosphorus which may contribute to algal blooms (Marshall, 1997).

An average urban Australian house uses up to 820 litres of water per day for indoor and outdoor use. Grey water is generated in the bathroom (180L), laundry (110L) and kitchen (55L). Each household varies from these averages depending on appliances and habits. While in a water-saving household in Europe approximately 50 to 55L of grey water are consumpted per day and person. (Dietmar Sperfeld, 2007). In the US, an average American contributes about 265 – 568 litres of wastewater per day, whereby grey water constitutes the largest proportion.

Grey water is about 99 percent water by weight and possesses a very low nutrient content and therefore it can be fairly easily treated to high quality water using simple techniques such as constructed wetlands, ponds, bio-films or activated sludge processes. For high-tech applications more sophisticated treatments, such as membrane filtration or activated carbon filters, may eventually be added (UNESCO/IPH, 2006).

Treated grey water can be put to particularly good use for agricultural irrigation (especially in water scarce regions), but may also be used for groundwater recharge, industrial or urban reuse or discharged into surrounding watercourses. Grey water can

also possibly be used for toilet flushing as well as washing machines (Dietmar Sperfeld, 2007). Grey water systems can be implemented decentralized as well as centralized. Decentralized grey water installations are, installations which process the grey water in the point of origin while central arrangements will bring the water from several housing units together and process them in a centralized point.

A study conducted by Ling *et al.* (2001) at an ecological sanitation pilot project involving nine houses which was situated at Hui Sing Garden, Kuching, Sarawak showed that the system was able to significantly improve the water quality in terms of dissolved oxygen (DO), total suspended solids (TSS), reactive phosphorus (RP), total phosphorus (TP), ammonium-nitrogen (AN), total nitrogen (TN), biochemical oxygen demand (BOD) as well as chemical oxygen demand (COD).

2.3 Oil degrading bacteria

Oil degrading bacteria can be characterized as bacteria species with the ability to utilize oil as their energy source or carbon source. This is made possible by the ability of those bacteria to produce enzymes such as lipases which can hydrolyze oil. There are vast amount of oil degrading bacteria which are readily available in the environment. Some have the ability to degrade mineral oil such as TPH (total petroleum hydrocarbon) (Bhattacharya *et al.*, 2003) while some have the ability to degrade organic oil such as vegetable oil, lard, margarine (Hasanuzzaman *et al.*, 2004) and etc. Research on the ability of certain bacteria isolated from different types of environments, are expanding through time.

Members of diverse genera have been reported to produce different types of lipolyitc enzymes. Sugimori *et al.* (2002) reported that *Acinetobacter sp.* Strain SOD-1 is an oil degrading bacteria with the capability to rapidly degrading salad oil. *Acinetobacter sp.* strain SOD-1 is a Gram-negative aerobic cocobacillus. Salad oil degradation by strain SOD-1 depended on the salad oil content. Strain SOD-1 had a high degradation rate at the content of less than or equal to 3000 ppm of salad oil. The bacterium degraded 85.0% of an initial 2000 ppm salad oil or suspension in 24 h at 28°C and pH 7. It was shown that Strain SOD-1 efficiently degraded salad oil over a wide temperature range (20-40°C) and the optimum temperature was found to be around 35°C.

In another work, Matsumiya *et al.* (2007) reported to successfully isolate 7 newly isolated strains from various environmental sources. The seven bacterial strain showed a high cell growth rate (OD660>1.0) in the screening medium. Strain *Burkholderia sp.* DW2-1 which showed the highest degradation rates was selected for further investigation. It was found that DW2-1 strain can be used for wastewater treatment without temperature control. The lipase activity of strain DW2-1 after 48h of cultivation was 1720U/l, whereas the commercially used oil degrading strain BN Clean (*B. subtilis* BN 1001) was below 7U/l. Matsumiya *et al.* (2007) also found that strain DW2-1 can degrade lipids at high concentrations in continuous cultivation, and that its lipid degradation ability is not affected by environmental microorganisms.

In a study conducted by Hasanuzzaman *et al.* (2004), a novel, oil degrading bacterium, *Pseudomonas aeruginosa* T1 was isolated from a hot spring in Hokkaido, Japan. The strain T1 has the ability to efficiently degrade different vegetable and animal fats or oils that also include edible-lipid wastes. Strain T1 had active lipase to hydrolyzed

products (DGs and MG), therefore, it is considered that the enzyme has random specificity towards each sn-position of the glycerol backbone of the substrates. This strain has the capacity to utilized FFA very efficiently, because most of the released FFA disappeared in cultures at 25°C or 30°C. Strain T1 produced high extracellular lipase activity when grown in the presence of salad oil or oleic acid. Similar results were obtained when salad oil was added at earlier phases of growth. Olive oil was also a good inducer of lipase. Irrespective of inducing compounds added to the medium, both lipase activity and bacterial growth were increased when lipids were added to the medium, but can also be utilized as energy and carbon sources.

In a similar study conducted by El-Bestawy *et al.* (2005), four bacterial species (*Pseudomonas sp, Pseudomonas diminuta, Pseudomonas pseudoalcaligens* and *Escherichia coli*) were isolated from wastewater drainage of Damanhour and Moharam Bek factories of the Extracted oils and Derivatives Company, Alexandria. The bacteria were found to have the ability to degrade oil and grease in the contaminated wastewater. The bacteria isolates were tested individually or in combinations using synthetic aqueous medium supplemented with 1% palm oil, incubated at 30°C, and agitated at 150 rev/min for 13 days. All the tested bacteria were able to degrade the palm oil completely and utilized the free fatty acids (FFA) as a carbon source.

Isolation of new oil-degrading bacteria from various environmental sources offers interesting implications because they are considered to be the most important group of biocatalysts used for a variety of different biotechnological applications (Hasanuzzaman *et al.*, 2004). It would be interesting to identify less expensive, stable lipases for industrial

application from newly examined microorganisms, especially those that might have unique selectivity and high activity.

2.4 Lipases

Lipases are important enzymes which have the ability to catalyze a number of reactions (Hasanuzzaman *et al.*, 2004). Lipases are esterases that are able to hydrolyze water insoluble esters such as long chain triacylglycerols. Lipases catalyze the formation of esters (esterification) and the exchange of esters (transesterification) when present in aqueous media. Lipases display a high degree of specificity and enantioselectivity for esterification and transesterification reactions.

Ecological concerns have favoured more extensive applications of lipases because lipase-catalysed reactions resemble more closely the pathways designed by nature for the metabolism of living things. The lipase discriminating ability encompasses features such as stereospecificity, selectivity and substrate specificity, which are much higher than those of inorganic catalysts (Hamid *et al.*, 2003).

Lipases are mainly obtained from microbial resources. Microbial lipases can be applied in detergents, manufacture of food ingredients and pitch control in the pulp and paper industry (Vargas *et al.*, 2004) as well as for medical purposes. Another potential usage of microbial lipases is in the bioremediation. Bioremediation is a technology that utilizes microorganisms for the restoration of contaminated environments. In certain circumstances, bioremediation can be enhanced by adding microorganism with specific metabolic functions – bioaugmentation. For example, lipase producing microbes can be added to an oil wastes contaminated area (Suzuki *et al.*, 2001).

The enormous potential of microbial lipases arises from the facts that they are quite stable and active in organic solvents and do not require cofactors. According to Hamid *et al.* (2003), more than 50 lipases have been identified, purified and characterized, which originated from natural sources such as animal, plants as well as microorganisms (native or genetically engineered). Bacterial lipases from particular bacterial species such as *Pseudomonas aeruginosa* is very interesting because these enzymes display special biochemical characteristics which is special compared to lipases produced by other microorganisms, such as their thermoresistance and activity at alkaline pH.

2.5 Molecular Techniques in Identification of Microorganisms

Molecular techniques had been proven to be efficient for the identification of oil degrading bacteria. Among the molecular techniques which are frequently utilized for this purpose are the polymerase chain reaction and phylogenetic analysis by using 16S rRNA gene sequence comparison.

2.5.1 Polymerase chain reaction

Polymerase chain reaction is a technique by which any piece of DNA can be quickly amplified (copied many times) without using cells. The DNA of interest is incubated in a test tube with a special kind of DNA polymerase, a supply of nucleotides, and short pieces of synthetic single stranded DNA that serve as primers for DNA synthesis (Campbell and Reece, 2002).

PCR can rapidly amplify; by which up to a billion fold of DNA molecules, yielding large amounts of specific genes for a host of application in molecular biology

(Madigan and Martinko, 2006). It is a powerful tool which is relatively easy to perform, extremely sensitive and specific, as well as highly efficient. During each round of amplification the amount of product doubles, leading to an exponential increase in the desired DNA. By using PCR, not only a large amount of amplified DNA can be produced just in a few hours, but that only a few molecules of target DNA need to be present in the sample to start the reaction.

PCR is extremely valuable for obtaining DNA for cloning genes and for sequencing purposes because the gene or genes of interest can be easily amplified if flanking sequences are known. PCR is also used routinely in comparative or phylogenetic studies to amplify genes from various sources. In these cases the primers are made to regions of the gene thought to be conserved throughout a wide variety of organisms.

For example, because 16S rRNA, a molecule used for phylogenetic analysis, has both highly conserved and highly variable regions, primers specific for the 16S rRNA gene from Bacteria or Archaea can be synthesized and used to survive the organisms in a habitat for a presence of species of each group. Moreover, if more specific primers are used, only certain subgroups within each domain can be targeted. This technique is in widespread use in microbial ecology and has revealed the enormous diversity of the microbial world, much of it still uncultured.

2.6 Phylogenetic analysis by using 16S rRNA gene sequence comparison

Relationships among bacteria have traditionally been examined using a variety of morphological (staining), biochemical and serological procedures and grouping together those bacteria that share the greatest number of traits. The resulting taxonomy, however,

does not necessarily reflect phylogeny, relationships by evolutionary descent (Campbell and Reece, 2002). Most microbiologists would prefer to have taxonomic schemes based on phylogeny since the grouped bacteria should share close genetic backgrounds and common phenotypes.

One of the methods which can be used to study the relationship among bacteria is the 16S and 23S rRNA gene sequence comparison. The DNA region between the 16S and 23S ribosomal RNA genes in bacteria (the intergene, IGR) is variable in length, and there are highly conserved sequences on each side (the 5'- end of the 16R rRNA gene and the 3'- end of the 23S rRNA gene) (Campbell and Reece, 2002).

Sequence analysis of 16S rRNA can be used to derive phylogenetic relationships in pure cultures of microorganisms (Madigan and Martinko, 2006). Likewise, sequence analysis of 16S gene amplified from a microbial community can paint a phylogenetic picture of that community. Alternatively, metabolic genes that encode protein unique to the metabolism of a specific organism can be the gene targets. However regardless of the gene chosen for PCR amplification, specificity of the primers is essential. With natural samples as sources of DNA, primer design is critical for obtaining PCR results that are unambiguous and readily interpretable.

3.0 Materials and Methods:

3.1 Isolation, media, and culture conditions

The isolation, media and culture conditions of this research was performed by following the methods described by Hasanuzzaman *et al.* (2004) and Matsumiya *et al.* (2007). Water samples were taken from the oil and grease trap of Kenanga College Grey Water Ecosan System. For each of the sampling process, 50 ml of water samples were obtained and were kept in a 100 ml Scotch bottle. The water samples were then transported to the laboratory immediately.

In this research, sampling activities had been started in mid November 2007. A total of 6 samplings were carried out throughout this research. The first sampling was carried out on the 15th November 2007. Subsequent samplings were conducted on the following dates: 19th November 2007, 6th December 2007, 4th January 2008, 8th January 2008 and 16th January 2008. For each sampling, a duplicate of samples were taken from both inside and outside of the oil and grease trap.

3.1.1 Enrichment culture technique

3.1.1 (a) Minimal salt medium (broth)

Water sample (1ml) was transferred aseptically into a 500 ml Erlenmeyer flask containing 100ml sterile minimal salt (MS) medium with the following components (g/L): KH_2PO_4, 3.0; Na_2HPO_4, 6.0g; NH_4Cl, 2.0g; NaCl, 5.0g; glucose, 8.0g; and $MgSO_4.7H_2O$, 0.1g. Sterilized olive oil (commercially obtained) (1%) was added to the flask and was incubated at room temperature with shaking at 100 rpm for 2 weeks. An aliquot of the culture (0.1 ml) was spread on PYA plates (1% peptone, 1% yeast extract, and 1.5% agar) containing emulsified olive oil (1% v/v) and was incubated at 37°C. Colonies that appeared after 24 hours of incubation were picked off and transferred on PYA with olive oil (1% v/v). Pure cultures were obtained by repeated streaking on fresh nutrient agar plates.

3.1.1 (b) Minimal salt medium (agar)

An aliquot of the water sample (0.1 ml) was spread directly on minimal salt agar plates (with the following components (g/L): KH_2PO_4, 3.0; Na_2HPO_4, 6.0g; NH_4Cl, 2.0g; NaCl, 5.0g; glucose, 8.0g; and $MgSO_4.7H_2O$, 0.1g) containing emulsified olive oil (1% v/v) and was incubated at 37°C.

3.1.2 Direct culture technique

3.1.2 (a) Direct inoculation

An aliquot of the water sample (0.1 ml) were spread directly on PYA plates containing emulsified olive oil (1% v/v) and were incubated at 37°C. Colonies that appeared after 24 hours of incubation were picked off and transferred on another PYA with olive oil (1% v/v). Pure cultures were obtained by repeated streaking on fresh nutrient agar plates.

3.1.2 (b) Diluted water sample

Water sample (1 ml) was transferred aseptically into a 500 ml Erlenmeyer flask containing 99 ml distilled water (total – 100 ml). An aliquot of the diluted water sample (0.1 ml) was spread on PYA plates (1% peptone, 1% yeast extract, and 1.5% agar) containing emulsified olive oil (1% v/v) and was incubated at 37°C. Colonies that appeared after 24 hours of incubation were picked off and transferred on PYA with olive oil (1% v/v). Pure cultures were obtained by repeated streaking on fresh nutrient agar plates.

3.2 Gram Staining

This method was carried out according to Madigan and Martinko (2006). Gram staining was performed on the bacterial colonies grown on the nutrient agar plates. Separate smears were prepared for bacterial isolates with different morphological characteristics. The smears were air dried and heat fixed. The slides were then labeled. Subsequently when the smears had dried, staining process was carried out. This process